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OTTAWA HULL K1A 0C9

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(19)(CA) **CANADIAN PATENT** (12)

(54) Solid Support for Immobilizing Nucleotide Sequences

(72) Georges, Fawzy , Canada

(73) Same as inventor

(57) 3 Claims

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ABSTRACT OF THE DISCLOSURE

The present invention relates to a support for the immobilization of nucleotide sequences. This support comprises a diol, a controlled pore glass particle and a phosphodiester linkage. The diol has its first hydroxyl group linked to the controlled pore glass particle through bonding to the phosphodiester linkage while its second hydroxyl group is free for linking to a nucleotide. The present invention also relates to a process for synthesizing nucleotide sequences. It first comprises immobilizing a nucleotide fragment to the above-mentioned support by linking nucleoside phosphoramidites to the free hydroxyl group of the diol. Then, a protected nucleotide segment is immobilized onto the support by tandem ligation to the immobilized fragment. Optionally, one or more nucleotide segments are ligated to the free end of the linked segment by tandem ligation. The synthesized nucleotide sequence is then deprotected and the support is submitted to the action of a restriction enzyme to recover the desired nucleotide sequence.

TITLE OF THE INVENTION

Solid support for immobilizing nucleotide sequences.

FIELD OF THE INVENTION

The present invention relates to a solid support useful in the immobilization of nucleotide sequences, preferably solid-phase DNA templates. The use of this support provides a novel process for DNA synthesis through which solid-phase DNA templates are used as a continuous source of supply for the synthesis of oligonucleotides of predetermined sequence and length.

BACKGROUND OF THE INVENTION

Synthetic DNA fragments immobilized on solid matrices have served as useful handles in a number of gene detection experiments, diagnostic interpretations, isolation and purification of mRNA mixtures and other genetic materials from complex soups of DNA, RNA and proteins. In addition, affinity columns containing only immobilized DNA were used successfully to isolate certain DNA binding proteins.

A number of procedures have been reported in the past few years describing various approaches to DNA immobilization. While the majority of these employ post-synthesis immobilization of synthetic DNA fragments, only two reports describe a during synthesis type of immobilization. The first approach described in (1988) Nucleic Acids Res., Vol. 16, 6232, utilizes the relatively

stable nucleoside-urethane linkage introduced in 1985 by Sproat and Brown and described in (1985) Nucleic Acids Res., Vol. 13, 2979-2987, whereas the second approach described in (1988) Anal. Biochem., Vol. 169, 104-108, is based on the teflon fiber support developed in 1984 by Lohrmann and described in (1984) DNA, Vol. 3, 122.

Although the urethane-linkage approach may be useful for somewhat shorter fragments, the preparation of immobilized fragments larger than 30 nucleotides in length is unsuccessful. This is presumably due to the apparent instability of this linkage under deprotection conditions for extended time periods. Moreover, a change in the appearance of the polymer is noted after filtration at the end of the deblocking procedure (12 hours at 50°C), suggesting possible breakdown of the silicic acid polymeric structure which would render it soluble in the concentrated ammonia solution.

The second approach, on the other hand, makes use of inert teflon fibers as the solid matrix for DNA synthesis and immobilization. Although this type of solid support is sufficiently stable under general deprotection conditions, it is somewhat difficult to handle due to its fine fibrous nature. Also, this type of support lacks the freedom of dynamic movement which ensures homogeneous surface exposure to the reaction medium.

In a DNA chain, individual nucleosides are linked together via phosphodiester groups, which are obviously resistant to relatively harsh alkaline media. In order to obtain solid support-oligonucleotide bridges with a similar degree of resistance to alkali, it would be highly desirable to develop solid supports through which a DNA chain could be covalently linked to resist aggressive environments.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a support for the immobilization of nucleotide sequences. It comprises a diol linked to a controlled pore glass particle through a phosphodiester bond.

Preferably, the phosphodiester bond will link the controlled pore glass particles to the diol through binding with both the glass particle and one hydroxyl group of the diol. The other hydroxyl group of the diol will be free to serve in nucleotide synthesis through coupling to commercial nucleoside phosphoramidites. More preferably, the diol will be an aliphatic diol.

The support of the present invention is also useful in the development of a process for synthesizing nucleotide sequences. This synthesis process comprises a first step in which a nucleotide fragment is immobilized to the above-mentioned support by linking nucleoside

phosphoramidites to the free hydroxyl groups of the diols found on the surface of the controlled pore glass particles. A protected nucleotide segment is then linked to this nucleotide fragment by tandem ligation. Optionally, additional nucleotide segments will be ligated to the end of the linked segment by tandem ligation to obtain a nucleotide sequence of suitable length.

Once the desired nucleotide sequence has been obtained, it will be deprotected according to methods known to those skilled in the art and submitted to the action of the appropriate restriction endonuclease followed by recovery of the desired nucleotide sequence.

The process through which the support of the present invention is prepared and the desired nucleotide segment attached thereto can be scaled up or down without any significant reduction in yields. Furthermore, since it requires no intermediary purification steps, the production of controlled pore glass supports derivatized using the process of the present invention reduction is foreseeable on a commercial level.

The loading capacity of the support of the present invention is estimated to be approximately 40 µmoles per gram of derivatized controlled pore glass, based on dimethoxytrityl cation color comparison with known standards.

Also, the support of the present invention is suitable for production of solid phase DNA templates that can be transcribed in vitro into sequence-specific RNA molecules.

A further advantage of the present invention is that the nucleotide sequences, such as DNA chains, synthesized on the novel derivatized solid matrix may be deprotected by a solution of sodium hydroxide. For example, a treatment using sodium hydroxide is sufficient to deprotect DNA strands of up to 75 nucleotides in length. Under these conditions, the oligonucleotides remain attached to the solid phase, the integrity of the nucleotide strand is unaffected and the physical appearance of the CPG support does not change. Therefore, this treatment offers the advantage of speed and high efficiency because of the fact that the synthesis and the immobilization may be done simultaneously, the support being able to retain the synthesized nucleotide strand during deprotection.

The phospho-CPG support of the present invention may be used in the synthesis of immobilized DNA fragments of various specific sequences, to establish basic models for affinity separation of DNA/RNA mixtures, for priming solid-phase enzymatic DNA synthesis, solid-phase DNA restriction as well as to purify commercial samples of a single-stranded DNA binding protein.

Hence, the process of the present invention provides for the first time a concept of DNA segment production which consists in using solid-phase DNA templates as a continuous source of supply for the synthesis of oligonucleotides of pre-determined sequence and length that are used frequently, such as universal primers and diagnostic probes or allele-specific oligonucleotides used in diagnosing various genetic disorders. Known examples are the detection of somatic ras mutations in peripheral blood cells from acute myelogenous leukemia patients, prenatal diagnosis of antitrypsin deficiency and B-thalassemia.

The present invention also introduces the basic elements for total automation of solid-phase enzymatic manipulations of DNA. In view of the work described in the present document, it should be possible to immobilize large segments of DNA, such as restriction fragments or whole plasmids, by tandem ligation to short synthetically-immobilized DNA fragments. Various enzymatic interconversions can then be performed and the DNA is subsequently released. At this juncture, automation of the dideoxy sequencing procedure of DNA is foreseeable since only one strand of the duplex is convalently attached to the solid matrix. This in turn should allow the displacement of the other strand by the sequencing primer in a similar fashion as described above. Further,

the methodology could lend itself generally to DNA strand separation of large duplexes where this can, at present, only be accomplished with much difficulty.

The present invention will be more readily illustrated by referring to the following description.

IN THE DRAWINGS

Figure 1a is a schematic representation of the process through which aliphatic diols are derivatized to compounds that can be phosphorylated.

Figure 1b is a schematic representation of the process through which a nucleotide strand immobilized on a preferred embodiment of the support of the present invention is copied by chain extension.

Figure 1c is a schematic representation of a solid-phase transcription of a DNA sequence immobilized on a preferred embodiment of the support of the present invention.

Figure 2 is a schematic representation of enzymatic reactions on a DNA segment attached to the support of the present invention.

Figure 3 represents the results of a polyacrylamide gel electrophoresis analysis conducted following primer extension reactions performed on a DNA segment attached to a preferred embodiment of the support of the present invention.

Figure 4 represents the results of a polyacrylamide gel electrophoresis analysis conducted following the binding of commercial rec A protein to an oligo-dc chain immobilized on the support of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a solid support comprising a CPG particle linked through a phosphodiester bond to one hydroxyl group of a diol which acts as a spacer arm. The other hydroxyl group of the diol is used in the synthesis of nucleotide sequences through coupling to commercial nucleoside phosphoramidites. Generally, the solid support of the present invention is prepared by using the following description.

The diol component is first derivatized with dimethoxytrityl chloride to yield a mixture of tritylated products that can be phosphorylated without prior purification as shown in Figure 1a. The phosphorylation procedure described by Narang et al. in (1980) Methods in Enzymology, 65, 610-620 has been found to be suitable, although phosphorylation procedures are well-known to those skilled in the art.

Once the desired diol, preferably an aliphatic diolhaving from 4 to 12 carbon atoms, has been tritylated

and phosphorylated, it is mixed with 1,2,4-triazol, dried and reacted before being reacted with a suspension of controlled pore glass support. Preferably, controlled pore glass support having a pore diameter of 240 A and which may be purchased from Pierce, will be employed. The resin is then reacted with the appropriate nucleoside phosphoramidites in order to immobilize short nucleotide fragments through ligation to the free hydroxyl group of the diol.

Once the desired CPG-immobilized DNA fragment is obtained, it is deprotected using a solution of sodium hydroxide. The time required for deprotection will depend upon the length of the nucleotide sequence to be deprotected.

Once this CPG-immobilized DNA fragment has been obtained, it can be extended through solid-phase primer extension reactions through the formation of a mixture containing the CPG-immobilized DNA fragment and the appropriate amount of a reaction buffer such as a Klenow reaction buffer. To this mixture may be added each of the four dNTPs and sufficient units of the Klenow large fragment. These techniques are well known to those skilled in the art. The reaction mixture is then mixed,

after which the enzyme is heat deactivated and the reaction supernatant analyzed by suitable techniques.

One may also wish to ligate fragments to immobilize single-stranded phosphorylated chains. In order to perform this operation, an aqueous solution containing the fragment to be ligated is reacted with the immobilized phosphorylated nucleotide chain and incubated. After the desired incubation period, a suitable ligase such as T4 DNA ligase is added and the mixture is again incubated.

If it is desired to release DNA fragments from the solid support, the solid material containing the desired DNA fragment is to be submitted to the action of the appropriate restriction endonuclease.

Moreover, the solid support of the present invention provides a process allowing quantitative conversion of the primer into extension products, irrespective of initial ratios of primer to template. Thus, the level of product formation is directly proportional to the amount of primer used, even at primer/template ratios of 20: 1 or higher, with the limiting factor being the total amount of dNTPs in the medium. A likely explanation for this remarkable efficiency of DNA amplification may be based on the

multiple rounds" type of mechanism which was observed earlier.

DESCRIPTION OF PREFERRED EMBODIMENTS

The preferred embodiments described below are introduced to illustrate rather than limit the scope of the present invention.

In all the DNA syntheses described below, a pre-sequence of 10 thymidine residues was incorporated at the 3' end of each synthesized chain (except where indicated otherwise). This stretch of thymidine served as an anchor for an oligodeoxyadenosine primer in which the last 3' nucleotide complemented the first base into the template following the thymidine stretch, for precise sequence alignment, which was used to copy the immobilized strand by chain extension as shown in Figure 1b. In this manner, the enzymatically extended chain provided means for indirect size determination of the immobilized fragment as well as confirming total deprotection of the chain while establishing its integrity and full biological activity.

It is interesting to note that one of the chain extension experiments yielded a strand which migrated much slower than expected in the sizing gel, indicating a chain length of almost double that of its corresponding immobilized template as shown in Figure 3. This

phenomenon may be attributed to the repetitive nature of the sequence $(dAdC)_{15}$ which may allow "DNA slippage", leading in turn to excessive elongation of the primer.

Derivatization of the aliphatic hydrocarbon diols

Dimethoxytrityl chloride (3.2 g, 9.5 mmoles) in 50 ml of dry pyridine was added dropwise to a solution of the diol, 1,5-pentanediol or 1,7-heptanediol (one equivalent) in pyridine (50 ml) with stirring. After 2 hours at room temperature t.l.c. examination of the reaction mixture revealed conversion of the diol into two new products corresponding to mono- and disubstituted diol. The solvent was evaporated under vacuum and the colored residue was washed with water to remove remnants of pyridine. The residue was dissolved in dichloromethane (50 ml) and washed twice with water (20 ml) and dried over sodium sulfate. Solvent removal gave a viscous syrup which was used in the next step without further purification.

CPG derivatization

The product mixture from the preceding experiment (2.0 g) mixed with 1,2,4-triazol (4.1 equivalents) were dried by repeated evaporation of dry pyridine under vacuum. Dissolved in pyridine to a final volume of 10 ml, the mixture was then cooled on ice and 4-chlorophenyl dichlorophosphate (2.4 g, 2 equivalents) was added dropwise with stirring while maintaining the

temperature at 5-10°C. After one hour, the mixture was added in small portions to a suspension of 1.0 g CPG support (vacuum dried in the presence of calcium sulfate desicant) in pyridine (2 ml) with occasional mixing. The reaction mixture was secured to a rotary evaporator, for gentle mixing for 16 hours at room temperature. The mixture was then diluted with acetonitryle and filtered. The filtrate was washed consecutively with water (2 X 10 ml), ammonium hydroxide (1 X 5 ml), methanol (2 X 10 ml), acetonitryle (2 X 10 ml) and dichloromethane (2 X 10 ml). The air dried CPG product was collected and a sample thereof (0.1 g) was treated with a 2% dichloroacetic acid solution in dichloromethane (2 ml) which gave the characteristic trityl cation color. Spectrophotometric measurements of the latter at 498 nm indicated a loading capacity of approximately 40 umoles/gram.

Deprotection of DNA fragments

A 0.5 M solution of sodium hydroxide was introduced gently into the synthesis cartridge containing the CPG-immobilized DNA fragment by means of a 5 ml syrenge. The cartridge was then mounted on a low speed motor (2 rpm) for gentle agitation which continued for 30-60 minutes, at room temperature, depending on the DNA chain length. The liquid was then withdrawn from the cartridge and the solid support was washed consecutively with water (10 ml), methanol (10 ml) and ether (5 ml).

The product was dried by a flow of nitrogen and collected into a microfuge tube.

Solid-phase primer extension reactions

The CPG-immobilized DNA fragment, 5.0 mg, in a 0.5 ml microfuge tube, was suspended in water, 20 ul, followed by addition of 100 ng of the appropriate primer and 4 ul of Klenow reaction buffer (10 x). To this was added 2 ul of each of the four dNTPs (0.5 mM) and the volume was adjusted to 40 ul by the addition of water. Two units of the Klenow large fragment were added and the tube was mounted on the slow-speed motor as in the preceding experiment. Mixing was allowed to continue for 30 minutes at room temperature. The enzyme was heat-deactivated (15 minutes at 65°C) and the CPG-template was centrifuged for 2 minutes at 5000 rpm. The supernatant was transferred to another tube for analysis by polyacrylamide gel electrophoresis. Results are shown in Figure 3.

Binding of commercial rec A protein to an immobilized oligo-dC chain

CPG-borne dC_{12} (100 mg, 4 umoles) in a 0.5 ml microfuge tube was suspended in TE buffer (100 ul) containing DTT (0.9 mM and $MgCl_2$ (25 mM). A commercial sample of rec A containing approximately 16 ug of protein (2 ul), diluted to 50 ul in the same buffer was introduced and the mixture was agitated as before for 30 minutes at

room temperature. The solid phase was allowed to settle to the bottom of the tube and the supernatant was transferred into another tube. The solid material was resuspended in the same buffer (100 ul) but containing ATP (30 mM) and agitation was resumed for 30 minutes. The supernatant was analyzed for the presence of the protein on an analytical Phast* system polyacrylamide gel (12.5%), run under native conditions. Results are shown in Figure 4.

Phosphorylation of immobilized DNA chains

An immobilized oligomer of 15 thymidine residues (15 mg) was suspended in 100 ul of buffer containing Tris hydrochloride, 50 mM (pH 7.4), magnesium chloride, 10 mM, dithiothreitol (DTT), 10 mM and ATP, 5 mM was treated with 2 units of the polynucleotide kinase enzyme. The mixture was agitated at room temperature for 60 minutes as described above. The solid phase was extracted with TE buffer (3 X 1 ml).

Ligation of DNA fragments to immobilized single-stranded chains

The phosphorylated chain from the preceding experiment was suspended and equilibrated in the same reaction buffer (100 ul). To this, an aqueous solution containing 300 pmoles (5 ul) of fragment AST, an oligo-dA fragment containing a Sau 3AI cohesive sequence at its 3' end and radiolabelled with ^{32}P (see Figure 2) was added.

* denotes a Trademark

B

After an incubation period of 60 minutes at 40°C, with agitation, T4 DNA ligase (10 units) was added and the mixture was incubated at 4-8°C with continuous agitation for 18 hours. This led to the incorporation of radioactivity into the solid material, indicating successful ligation. The solid material was extracted repeatedly with TE buffer containing sodium chloride (1M) until no more radioactivity was released. Subsequent treatment of this material with Sau 3AI released the radioactivity back into the medium, while the rest of the DNA chain remained immobilized but had no detectable radioactivity. The difference in radioactivity lost to the solid support was calculated to correspond to approximately 120 pmoles of fragment AST. These reactions were carried out by sequential washing and resuspension of the solid support in the appropriate buffer for each enzyme, thus reducing considerably the time required for each reaction by eliminating a number of isolation and purification steps.

Release of DNA fragments from the solid support by restriction endonucleases

The solid material obtained in the preceding experiment was suspended in 100 ul of fresh Klenow buffer containing the oligodeoxyadinosine primer (200 pmoles) and the four dNTPs (final concentration of 0.2 mM in each dNTP). To this reaction mixture was added 2 units of the

Klenow enzyme. Agitation for 15 minutes at room temperature was followed by gentle withdrawal of the buffer and resuspension in 100 ul of the Sau 3AI reaction buffer. The enzyme (15 units) was then introduced and agitation was resumed for 90 minutes at room temperature, after which time an estimated 80% of the radioactivity was released back to the supernatant.

Transcription of solid phase DNA templates into sequence-specific RNA.

The present invention may also be used to produce solid phase DNA templates that can be transcribed in vitro into sequence-specific RNA molecules. The approach is based on the elegant procedure described by Milligan et al. in (1988) Nucleic Acids Res., Vol. 16, 6232, for the in vitro transcription of synthetic DNA templates in solution using T7 RNA polymerase.

Thus, by incorporating the necessary 17 nucleotide long T7 promoter element in the start of DNA synthesis-immobilization, and following essentially the same transcription procedure, solid phase transcription of the DNA sequence downstream from the promoter was achieved. High levels of RNA transcripts were consistently obtained, supporting the earlier observation. It therefore seems that solid phase DNA templates used for either RNA transcription or DNA replication are required only in catalytic amounts.

Claims to the invention follow.

CLAIMS

1. A support for the immobilization of nucleotide sequences, said support comprising:

- a diol,
- a controlled pore glass particle, and
- a phosphodiester linkage

said diol having its first hydroxyl group linked to said controlled pore glass particle through bonding to said phosphodiester linkage and having its second hydroxyl group free for linking to a nucleotide.

2. A support according to claim 1, wherein said diol is an aliphatic diol having between 4 and 12 carbon atoms.

3. A process for synthesizing nucleotide sequences which comprises:

- immobilizing a nucleotide fragment to the support according to claim 1 by linking nucleoside phosphoramidites to the free hydroxyl group of said diol;
- immobilizing a protected nucleotide segment onto said support by tandem ligation of said segment to said immobilized fragment, and optionally ligating additional nucleotide segment to the free end of the linked segment by tandem ligation;
- deprotecting the synthesized nucleotide sequence;
- submitting said support to the action of a restriction enzyme and recovering the desired nucleotide sequence.

$$HO\ (CH_2)_n\ OH + (CH_3O)_2TrCl$$

$$HO\ (CH_2)_n\ OTr(CH_3O)_2 \quad 1$$

+

$$(CH_3O)_2TrO\ (CH_2)_n\ OTr(CH_3O)_2 \quad 2$$

$n = 5$ or 7

FIGURE 1a

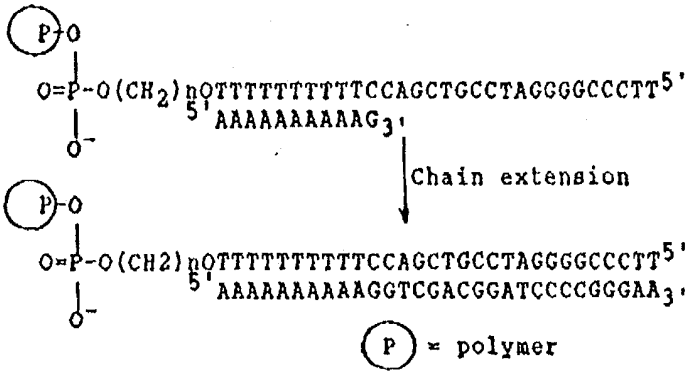


FIGURE 1b

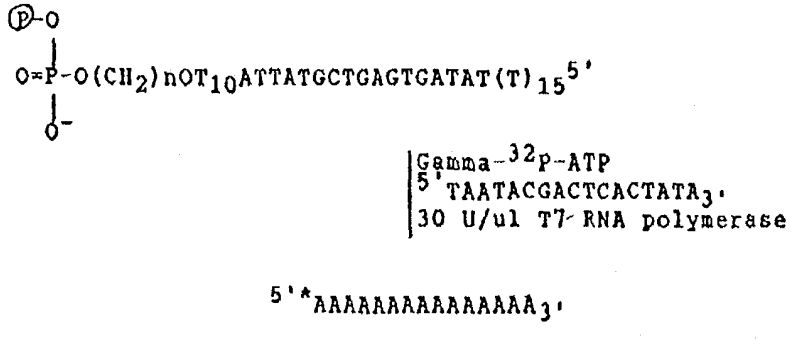
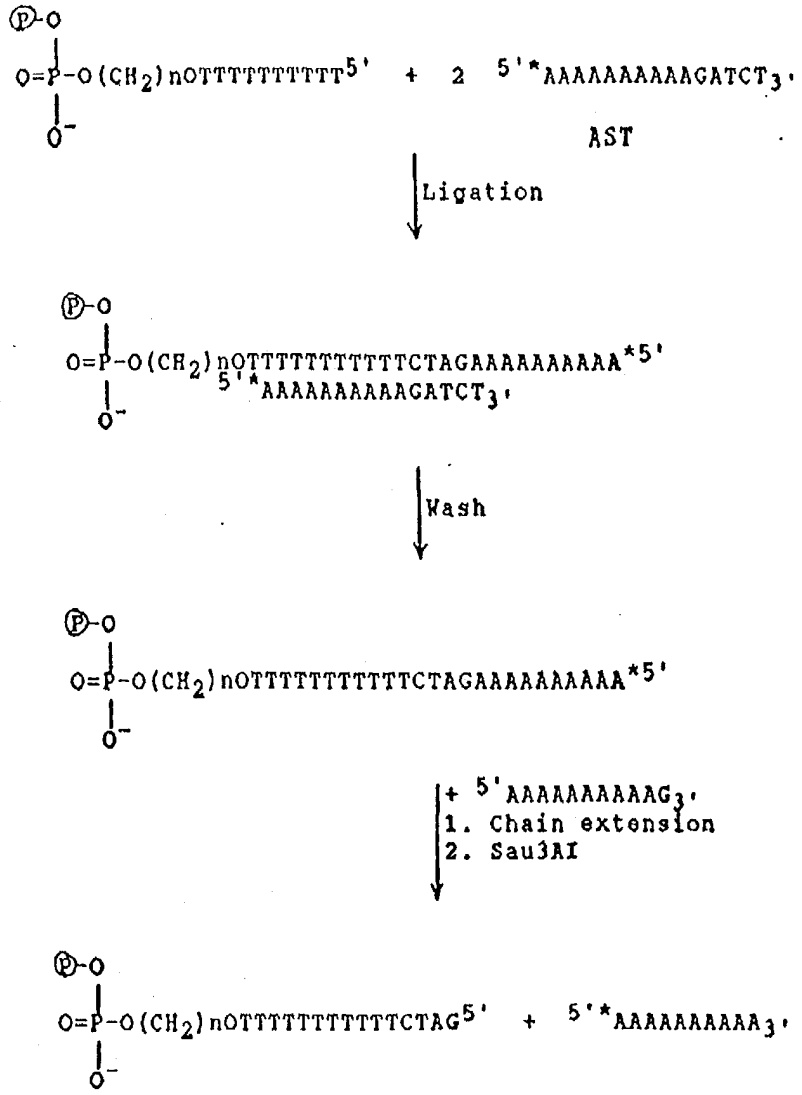
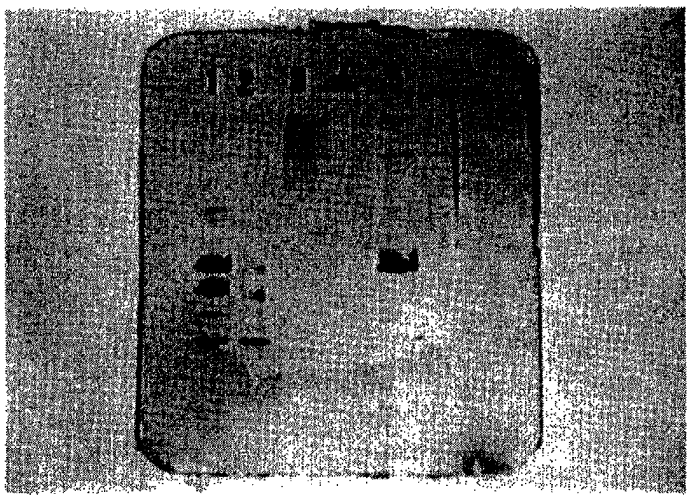


FIGURE 1c



Scheme IV: * = radiolabel

FIGURE 2



Lane 1: Commercial Rec A Protein
2: First wash through the column
3+4: Subsequent washes
5: Elution of the protein from the support

FIG. 3

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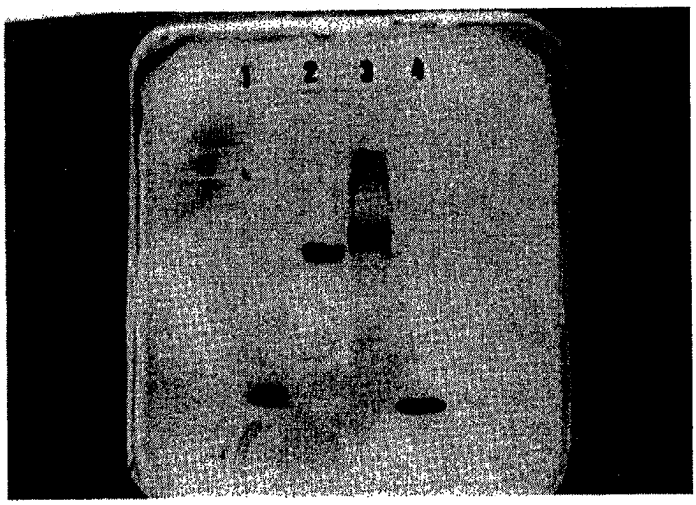

Lane 1: Marker 15mer
2: Marker 71mer
3: Reaction product
4: Extension primer A15 (control)

FIG. 4